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Sensitive time-resolved fluoroimmunoassay for simultaneous detection of serum thyroid-stimulating hormone and total thyroxin with Eu and Sm as labels

Feng-Bo Wu,* Shi-Quan Han, Tao Xu, and You-Feng He

Department of Isotope, China Institute of Atomic Energy, P.O. Box 275-39, 102413, Beijing, People's Republic of China

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Abstract

Based on a novel cocoating strategy and dissociation enhancement lanthanide fluorescence immunoassay technique, a sensitive time-resolved fluoroimmunoassay (TRFIA) has been developed for simultaneous quantification of human serum thyroid-stimulating hormone (TSH) and thyroxin (T4) in a one-and-the-same assay procedure. The new cocoating strategy for preparing highly active surface anti-TSH and anti-T4 monoclonal antibodies (McAbs) was performed by a three-step protocol. Namely, anti-TSH McAb at high concentration (10 μg/ml) and extensively biotinylated bovine serum albumin (BSA) at low concentration (0.5 μg/ml) were coated on microwells by passive adsorption, then streptavidin was captured by the surface BSA–biotin, and finally biotinylated anti-T4 McAb was immobilized by the remnant binding sites of the bound streptavidin. In the present TSH/T4 TRFIA, both sandwich- and competitive-type configurations were involved, and Eu^{3+} and Sm^{3+} were used as labels for TSH and T4 detection, respectively. The method showed rapid kinetics; the equilibrium was reached within 30 min at 37 °C due to the use of high concentrations of reaction reagents, rapid agitation, and small reaction volume. The lower limits of detection of the method were 0.028 mIU/L for TSH and 4.1 nmol/L for T4 with 20 μL of sample volume. The assay ranges for TSH and T4 were 0.21–80.00 mIU/L and 20–300 nmol/L, respectively. The correlation between the TSH/T4 values obtained by the present TSH/T4 TRFIA and those obtained by commercial chemiluminescence immunoassay was satisfactory.

Multianalyte immunoassay (MAIA),[1] of which two or more analytes of one sample are measured simultaneously in a single assay, is a long-cherished goal for clinical chemists due to its advantages of work simplification, increased throughput, and reduced overall cost per test [1]. Accordingly, MAIA has a high application potential in various areas such as virology, molecular biology, and microbiology, as well as for routine diagnosis. Up to now, different principles have been proposed for performing MAIA; generally they can be classified into two main formats: that based on multiprobes and that based on spatially separated test zones. Of the two formats, the multiprobe approach relies on the discrimination and detection of specific signals from different probes.

In addition to the early use of radioisotopic labels [2,3], several nonradioisotopic labels have been tried to design multiprobe-based MAIA. Attempts to use enzymes as labels led to only limited success due to the significant spectral overlapping of the end products and the difficulty in setting identical conditions (e.g., the pH value) optimal for all the enzyme activities [4]. Immunoassay using fluorescent or chemiluminescent-substrate as label offers only a possibility to improve such MAIA

* Corresponding author. Fax: +86-1069357195.
E-mail addresses: wufb@sina.com, wufb22@21cn.com (F.-B. Wu).

[1] *Abbreviations used:* DELFIA, dissociation enhancement lanthanide fluorescence immunoassay; TRFIA, time-resolved fluoroimmunoassay; McAb, monoclonal antibody; SA, streptavidin; BSA, bovine serum albumin; CLIA, chemiluminescence immunoassay; MAIA, multi-analyte immunoassay; ICP-MS, inductively coupled plasma mass spectrometry; BAC-NHS, biotinamidocaproate *N*-hydroxysuccinimide ester; PBQ, *p*-benzoquinone; DMF, *N,N*-dimethyl form-amide; T4, thyroxin; TSH, thyroid-stimulating hormone; DTTA, *N*′-[*p*-isothiocyanatobenzyl]diethylene triamine-N^1,N^2,N^3,N^4-tetraacetate; PBS, phosphate-buffered saline; RT, room temperature; ANS, 8-anilinonaphthalene sulfonate; EDC, 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide.

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[5,6]. The well-established CLIA has been applied to MAIA based on signal spatial discrimination [7], but in general such an assay is not suitable for multiprobe-based MAIA since the light emissions of different luminophores are often of similar wavelengths [8]. MAIA with different DNA fragments as labels was also investigated to detect three protein analytes [9]. In this design, the problem of signal overlapping was overcome since different DNA molecules can be easily separated before quantification. The sensitivity of the assay was very high because of the great sensitivity of PCR technology. However, the method was too technically demanding because of the complicated reagent-processing steps and the strict conditions for PCR amplification. Theoretically, a fluorometry-based assay may be particularly well suited for MAIA since it could combine several parameters simultaneously for signal discrimination, such as excitation and emission wavelength, decay time, and polarization. However, until now conventional fluorescent labels did not succeed in MAIA owing to their high backgrounds, short decay times and broad spectra, which make it difficult to distinguish the emission bands from each other. Up to the present, the above investigations have not found wider acceptance in clinic routines.

Meanwhile, lanthanides or their chelates have become the choice of label for developing MAIA. Some lanthanide ions emit strong fluorescence (i.e., Eu^{3+}) when chelated with appropriate organic ligands [10]. The fluorescence arising from the lanthanide chelates has the advantages of high quantum yield, long decay time, exceptionally large Stoke's shift, and narrow emission peaks. By exploiting these features, specific chelate fluorescence can be efficiently distinguished from the natural fluorescence or the scattered light, and the fluorescence coming from different lanthanides can also be easily discriminated due to their difference in decay time and emission wavelength. These features make the lanthanide (or its chelate) preferable to any other probes for developing multilabel-based MAIA. Of the 15 lanthanide ions, Eu^{3+}, Sm^{3+}, and Tb^{3+} have been used in dual-label [11–15] or triple-label MAIAs [16]. Based on a novel cofluorescence enhancement principle, quadruple-label TRFIA has also been constructed for detection of four analytes [17]. Nowadays, dual-label TRFIA kits (i.e., HCG/AFP DELFIA, t-PSA/f-PSA DELFIA; Wallac Oy) and the corresponding instruments are commercially available. Different types of analytes such as protein antigens [11], haptens [12], antibodies [13], viruses [14], NK cell cytotoxicity [15], and DNAs [16] have been measured by the time-resolved fluorometry-based multianalyte analysis. More recently, MAIAs using stable luminescent chelate labels have attracted wide attention [18–20]; it can be expected that these and upcoming studies in this area will open up new prospects for the development of MAIA and other types of bioanalysis.

Serum TSH and T4 measurement by immunoassay is the widely used screening test for biochemical assessment of thyroid status. In our previous work, we have developed T4 TRFIA [21,22] and a highly sensitive serum TSH time-resolved immunofluorometric assay [23]; serum TSH was also analyzed by immunoassay that employed an inductively coupled plasma mass spectrometry (ICP-MS) technique for Eu detection [24]. The purpose of present study was to develop a TRFIA for simultaneous detection of serum TSH and T4 with Eu and Sm as labels. To obtain a high sensitivity for TSH detection and to make the Sm signal strong enough for precise measurement, a novel cocoating strategy was proposed to prepare highly active surface anti-TSH and anti-T4 antibodies with the use of biotin–SA interaction. The present TSH/T4 TRFIA is sensitive, rapid, and simple to perform. For comparison, the TSH and T4 concentrations in 39 human serum samples were measured by the present TSH/T4 TRFIA; a good correlation was observed between this method and the Corning CLIA ($r = 0.997$ for TSH; $r = 0.921$ for T4). The development details and the assay performance features of the TSH/T4 TRFIA are described.

Materials and methods

Chemicals and buffers

Anti-T_4 McAb (McAb-6901) and anti-TSH McAbs (McAb-03, McAb-04, and McAb-05) were obtained from Medix Biochemica (Finland). Microtitration strips were products of NUNC (Denmark). DTTA-Eu^{3+} (*N*′-[*p*-isothiocyanato-benzyl]-diethylene-triamine-N^1, N^2, N^3, N^4-tetraacetate-Eu^{3+}) and DTTA-Sm^{3+} were from Tianjin Radio-Medical Institute (Tianjin, China). BSA was from Shenzhen JingMei Biotech (Shenzhen, China). SA, biotinamidocaproate *N*-hydroxysuccinimide ester (BAC-NHS), activated charcoal, TSH, and T4 were products of Sigma Chemical, (St. Louis, MO USA). *p*-Benzoquinone (PBQ) was from the Second Chemical Factory of Beijing (Beijing, China). Wallac T4 DELFIA kit and hTSH Ultra DELFIA kit were used to calibrate the TSH/T4 standards of present TSH/T4 TRFIA, and the assays were performed exactly according to the instructions enclosed in the kits.

The assay buffer was 100 mmol/L Tris–HCl, pH 8.4, containing 0.25 mg/ml ANS, 4.0 mg/ml sodium salicylate, 0.1% BSA, 0.04% NaN_3, 0.9% NaCl, 0.08% Tween 20, and 0.5% normal mouse serum. The coating buffer was 100 mmol/L sodium carbonate buffer (pH 9.3), containing 0.9% NaCl and 0.04% NaN_3. The washing buffer was 10 mmol/L Tris–HCl (pH 8.0) containing 0.04% Tween 20 and 0.9% NaCl. TSA buffer for elution of the Eu^{3+}- or Sm^{3+}-labeled reagents was 50 mmol/L Tris–HCl, pH 7.8, containing 0.9% NaCl and 0.05%

NaN_3. The enhancement solution for Eu^{3+}/Sm^{3+} dissociation and fluorescence enhancement was prepared according to Hemmilä et al. [25].

Instrumentation

The chromatographic separation system was a product of Bio-Rad, mainly including Model EP-1 Econo Pump and Model EM-1 Econo UV monitor. The VICTOR^2 multilabel counter,1296-003 DELFIA Plateshake, and 1296-026 DELFIA Platewash were products of Perkin–Elmer Wallac. The UV absorbance was measured by UV–vis spectrophotometer (Cary 50 mode; Varian, USA). Manual pipetting was done with disposable plastic tips and Finnpipette (Labsystem Oy, Helsinki, Finland).

Samples and comparison method

All samples were kindly provided by 301 Hospital (Beijing, China) with the TSH and T4 values measured by ACS-180 CLIA (Corning). The TSH values in 11 hypothyroid samples and 19 euthyroid samples were measured by the present TSH/T4 TRFIA. At the same time, the T4 values in the above 30 samples and in 9 hyperthyroid samples were also measured. All the patients were diagnosed on the basis of characteristic clinical features and confirmed by laboratory tests.

Preparation of human serum-based TSH/T4 standards

TSH-free human serum was prepared by treating the pooled serum from hyperthyroid patients with activated charcoal, as described by Mitsuma et al. [2]. The same procedure was applied to remove T4 from the normal human serum. The TSH concentration in the charcoal-treated serum was taken as zero, as no response was detectable by the hTSH Ultra DELFIA kits (analytical sensitivity: 0.001–0.002 mIU/L). The six TSH/T4 standards were prepared by adding different amounts of TSH and T4 in hormone-free serum, and the TSH/T4 concentrations were calibrated with Wallac hTSH Ultra or T4 DELFIA kits. In order to decrease the slight cross talk between the Eu/Sm signals and make the TSH/T4 concentration in the standards closer to most of the physiological conditions, the lower TSH concentration in the standards was set to correspond to the higher T4 concentration in the standards. The concentrations of TSH (mIU/L)/T4 (nmol/L) in the six standards were determined as 0/300, 0.21/150, 0.54/100, 2.91/50, 19.00/20, and 80.00/0.

Preparation of BSA–T4 conjugate

Twenty-five milligrams of PBQ in 1.5 ml of ethanol was added to 6 ml of PBS (0.25 mol/L, pH 6.0) containing 400 mg BSA. The mixture was incubated for 2 h with shaking. Free PBQ was removed by passage through a Sephadex G-50 column (30 × 1.5 cm, fine) with 0.9% NaCl solution as eluent. Twenty milliliters of the PBQ-activated BSA was collected. After this, 3 mg of 3,5,3′,5′-tetraiodo-L-thyronine in 0.1 mol/L sodium carbonate buffer (pH 9.6) was added to the activated BSA solution, the mixture was reacted for 12 h at RT. The BSA–T4 conjugate was separated from the excess T4 by applying the mixture to Sephadex G-50 column (30 × 1.5 cm, fine) and eluting the column with 50 mmol/L a carbonate buffer (pH 9.0). Twenty-five milliliters of conjugate was collected.

Biotinylation of BSA to different extents

To five glass bottles containing ~6 mg of BSA in 2 ml of carbonate buffer (50 mmol/L, pH 9.5), we added different amounts of BAC-NHS in order to obtain five BSA–biotin conjugates with different biotinylation extents. The volumes of BAC-NHS solution (in DMF, 8 mg/ml) added to the above five bottles were 4, 10, 20, 40, and 100 µl, respectively. The biotinylation protocol was the same as that described in our previous work [22]. After purification, the five BSA–biotin conjugates obtained were coded as BSA–biotin-4, BSA–biotin-10, BSA–biotin-20, BSA–biotin-40, and BSA–biotin-100, corresponding to the volume of the biotinylating reagent added.

The cocoating protocol for preparing highly active surface anti-TSH and anti-T4 McAbs

The cocoating protocol used in the present TSH/T4 TRFIA involved three steps. (1) 100 µl of coating buffer containing 10 µg/ml anti-TSH McAb (McAb-05) and 0.5 µg/ml BSA–biotin-40 was added in microwells and incubated for 12 h at RT. This step allowed the anti-TSH McAb (McAb-05) and BSA–biotin conjugate to be passively adsorbed on the well surface. (2) The microwells were washed twice, and 100 µL of TSA buffer containing 1% BSA and 0.8 µg/ml SA was added and agitated for 6 h at RT. SA was specifically captured by the surface biotin moieties. (3) After the wells were washed twice, 100 µl of TSA buffer containing 1 µg/ml biotinylated anti-T4 McAb and 1% BSA was added and agitated for 6 h at RT. The biotinylated anti-T4 McAb was bound by the surface SA. A schematic diagram of the surface anti-TSH and anti-T4 McAbs prepared by this cocoating procedure is shown in Fig. 1.

Optimization of the cocoating procedure

In order to test the utility of above five BSA–biotin conjugates for anti-T4 McAb immobilization, we studied the influence of the coating concentration of the five

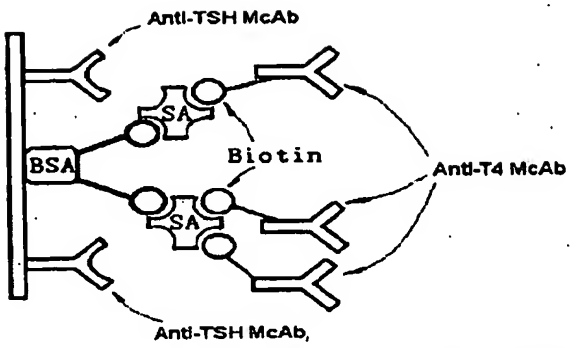

Fig. 1. Schematic representation of the surface anti-TSH and anti-T4 McAbs prepared by the proposed cocoating protocol.

conjugates on the activity of surface anti-T4 antibody. The five BSA–biotin conjugates were diluted with coating buffer to concentrations ranging from 0.176 to 10 μg/ml. The procedure for preparing surface anti-T4 McAb was the same as described above except that anti-TSH McAb was omitted from the first coating step. Namely, 100 μl of BSA–biotin solution at different concentrations was added to microwells and incubated for 12 h at RT. After the well were washed twice, 100 μl of TSA buffer containing SA (1 μg/ml) and 1% BSA was added and agitated for 6 h at RT. After the microwells were washed twice, 100 μl of TSA buffer containing 1 μg/ml biotinylated anti-T4 McAb and 1% BSA was added and agitated for 6 h at RT.

The activity of surface anti-T4 antibody prepared by above procedure was evaluated as follows. The microwells in five strips (12 microwells per strip) that were coated with anti-T4 antibody via the five different BSA–biotin conjugates were washed twice. 25 μl of T4-free serum and 50 μl of assay buffer containing ~100 ng of T4–BSA–Eu^{3+} [21] were added. The mixture was incubated for 1 h at RT with slow shaking. The wells were washed six times and the Eu^{3+} fluorescence was measured using the VICTOR^2 multilabel counter by a routine dissociation–enhancement protocol. The fluorescence intensity, which reflects the activity of the surface anti-T4 McAb, was plotted against the coating concentration of each BSA–biotin conjugate, as shown in Fig. 2. An identical procedure was performed to prepare another batch of surface anti-T4 McAb except that the SA concentration was changed to 0.5 μg/ml. The activity of the surface anti-T4 McAb was tested in the same way as described above.

The anti-TSH McAb in the first cocoating step will compete with BSA–biotin in adsorbing on the limited inner-well surface. To assess the influence of the concentration of anti-TSH McAb on the activity of surface anti-T4 McAb, we first prepared different surface antibodies via the above cocoating procedure with the concentration of BSA–biotin-40 kept unchanged (0.5 μg/ml) and the concentration of anti-TSH McAb varied

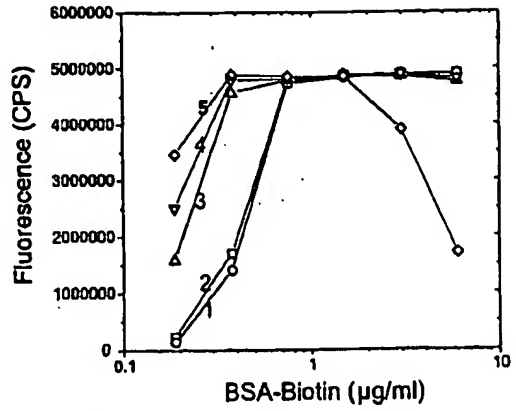

Fig. 2. The effects of the coating concentration of different BSA–biotin conjugates on the activity of surface anti-T4 McAb. Curves 1–5 were obtained by using BSA–biotin-4 (○), BSA–biotin-10 (□), BSA–biotin-20 (△), BSA–biotin-40 (▽), and BSA–biotin-100 (◇), respectively. The concentration of streptavidin in the second coating step was 1 μg/ml.

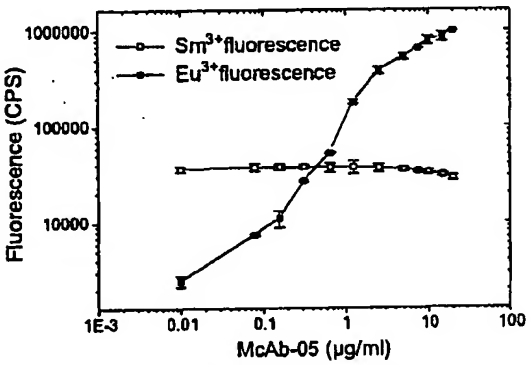

Fig. 3. The effects of the coating concentration of anti-TSH McAb on the activity of surface anti-TSH (■) and anti-T4 (□) McAbs in the proposed cocoating method. The concentration of the BSA–biotin used in the first coating step was kept constant (0.5 μg/ml of BSA–biotin-40). Error bars indicate twice the standard deviation of duplicate measurements.

from 0.01 to 20 μg/ml. The last two steps of the cocoating protocol were the same as described above. After the wells wer washed twice, 25 μl of standard F (TSH, 80 mIU/L; T4, 0 nmol/L) was added, followed by addition of 50 μl of assay buffer containing ~300 ng of europium-labeled anti-TSH McAb and ~600 ng of T4–BSA–Sm. The mixture was agitated for 2 h at RT. After the wells were washed six times, the fluorescence of Eu and Sm was measured by a routine dissociation–enhancement procedure and plotted versus the coating concentration of anti-TSH McAb (Fig. 3).

Eu and Sm labeling

The details of labeling anti-TSH McAbs (McAb-03 and McAb-04) with Eu have been described elsewhere

[23], and the same procedure was applied to label BSA–T4 conjugate with Sm except the molar ratio of DTTA–Sm/BSA–T4 was raised to 250 in order to get extensive labeling.

Serum TSH/T4 TRFIA

Microwells coated with anti-TSH and anti-T4 McAbs were washed twice with washing buffer. Twenty microliters of TSH/T4 standards or samples was pipetted in duplicate into the microwells, followed by the addition of 50 µl of assay buffer containing 500 ng of T4–BSA–Sm and 150 ng of each of the europium-labeled anti-TSH McAbs (McAb-03-Eu and McAb-04-Eu). The mixture was incubated for 40 min with rapid shaking at 37 °C. The wells were washed six times, 100 µl of enhancement solution was added, and the wells were agitated for 5 min. The fluorescence arising from Eu and Sm was measured using the Eu/Sm dual-label time-resolved fluorescence measurement program set in the VICTOR^2 multilabel counter. The conditions for Eu measurement were as follows: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 0.40 ms; window time, 0.40 ms; cycling time, 1.0 ms. While for Sm measurement the excitation wavelength and the cycling time were not changed, the delay time, emission wavelength, and window time were set at 0.050 ms, 642 nm, and 0.10 ms, respectively. The standard curves for the present TSH/T4 TRFIA were obtained by plotting the Eu fluorescence versus TSH concentration or by plotting the calculated B/B_0 value versus the T4 concentration, as shown in Figs. 4 and 5.

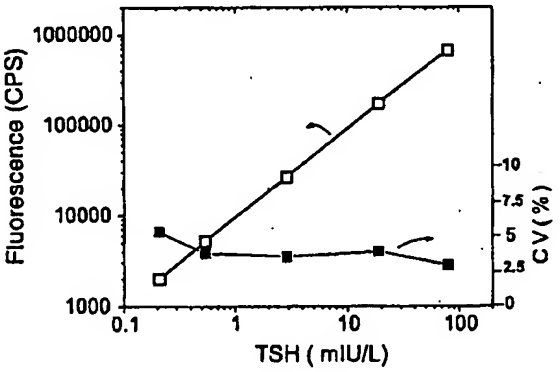

Fig. 4. Standard curve and the within-assay precision profile ($n = 10$) for the TSH assay in the present TSH/T4 TRFIA (background subtracted).

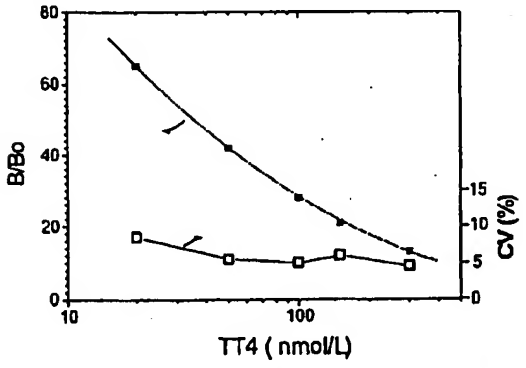

Fig. 5. Standard curve and the within-assay precision profile for T4 assay ($n = 10$) in the present TSH/T4 TRFIA. The Sm fluorescence for six standards was 51,200 (T4, 0 nmol/L), 33,280 (T4, 20 nmol/L), 21,500 (T4, 50 nmol/L), 14,340 (T4, 100 nmol/L), 10,750 (T4, 150 nmol/L), and 6656 (T4, 300 nmol/L).

Results and discussion

Preparation of BSA–T4 conjugate with the PBQ method

The T4–BSA conjugate, as a 2,5-substituted hydroquinone, was prepared by two successive addition–oxidation reactions with the use of PBQ as the "bridge" molecule. BSA was first reacted with PBQ at acidic pH, and 2-substituted hydroquinone was formed via the nucleophilic attack of the amino or hydroxyl group of BSA molecule. After this, hydrogen was eliminated by reaction with a second molecule of PBQ, giving a 2-substituted quinone derivative; BSA was thereby converted to a reactive intermediate. In the second step, the amino group in the T4 molecule attacks the quinone in the 5 position at alkaline pH, resulting in T4–BSA conjugate. This method is simpler than the previously described EDC-based protocol [21] because the tedious procedure for T4 methylation was omitted and the T4/protein ratio in T4–BSA conjugate is more controllable. The prepared BSA–T4 conjugate has a strong absorbency at 340 nm, a wavelength slightly red-shifted from the maximum absorbency (325 nm) of the free T4 molecule.

Evaluation of the cocoating procedure

Currently, direct passive adsorption of two or more binders is still the routine method for developing MAIA [9,11,17]. When only the sandwich assay is employed in the MAIA, it is usually desirable to prepare surface binder (e.g., antibody) with high activity so as to get a high sensitivity. Due to the difficulty in optimizing the assay system, the reports of MAIA including both competitive and sandwich-type configurations are still few. In this work, preparing highly active surface anti-TSH and anti-T4 antibodies represented one of the main challenges, because both the sensitive TSH analysis and the relatively low specific activity of the Sm label require the use of highly active surface anti-TSH and anti-T4 antibodies. In our initial experiments, we tried to coat

the anti-TSH and anti-T4 antibodies with the direct physical adsorption method, but we failed to obtain surface antibody that could exhibit enough activity for both TSH and T4 assay. As expected, the activity of the surface anti-TSH McAb was reduced as the concentration of the anti-T4 McAb was increased in the antibody mixture and vice versa, due to the adsorbing competitiveness of these two antibodies. Moreover, when the anti-T4 McAb was used at large excess to anti-TSH McAb (e.g., at a molar ratio of 9/1), the activity of the surface anti-T4 McAb was still not abundant enough to yield Sm signals strong enough for T4 assay (generally <8000 cps for zero T4 standard). Meanwhile, the activity of the surface anti-TSH McAb was too low to achieve sensitive TSH detection because of the low fraction of the anti-TSH McAb in the coating buffer.

The biotin–SA interaction has been used to improve the activity of surface anti-T4 McAb with a three-step protocol [22]: BSA–biotin adsorption, SA binding, and the specific immobilization of the target biotinylated anti-T4 McAb. Further experiments of this study demonstrated that BSA–biotin conjugates with different biotinylation extents can all be used to prepare surface anti-T4 McAb with almost identical maximum activity, although a higher concentration of the BSA–biotin, in case it bears fewer biotin moieties, was required (Fig. 2). Also, highly biotinylated BSA can be used at a very low concentration (e.g., BSA–biotin-40 or BSA–biotin-100 at 0.4 μg/ml) to obtain the same maximum activity of the surface anti-T4 McAb. Increasing the SA concentration, e.g., from 0.5 to 1 μg/ml, did not further increase the maximum activity of the surface anti-T4 antibodies, but it allowed using a higher concentration of BSA–biotin. More impressively, when the anti-TSH McAb was included in the first step of the cocoating protocol, even at a concentration as high as 10 μg/ml, the competitive adsorption between anti-TSH McAb and BSA–biotin did not obviously decrease the activity of surface anti-T4 McAb (Fig. 3). About ~80% of the maximum activity (that obtained in the absence of the anti-TSH McAb) of the surface anti-T4 McAb was reached when the concentration of anti-TSH McAb and BSA–biotin-40 in the first adsorption step was 10 and 0.5 μg/ml, respectively. Based on above observations, we proposed a new cocoating strategy. In the cocoating protocol, the microwells were first coated with anti-TSH McAb (10 μg/ml) in the presence of trace amounts of extensively biotinylated BSA (0.5 μg/ml BSA–biotin-40), and then SA was bound by the surface biotin moieties. Finally, biotinylated anti-T4 McAb was specifically immobilized via the remnant binding sites of the surface SA. This cocoating strategy not only guaranteed effective adsorption of anti-TSH McAb because of its high excess above the BSA–biotin conjugate in the first adsorption step, the activity of the surface anti-T4 McAb was also improved compared to that prepared by the direct passive adsorption. This improvement of the surface antibody activity can be, at least partly, explained by the following considerations. First, the anti-T4 McAb prepared by the proposed cocoating protocol was kept apart from the well surface by two proteins (BSA and SA) and two "spacers" introduced by the two biotinylation reagents (Fig. 1). This makes the anti-T4 McAb and anti-TSH McAb positioned at two different molecular layers above the well surface (the anti-T4 McAb can extend more deeply in the solution), the interference between the two surface antibodies in binding their analyte was thus reduced. Second, the large difference of p*I* between IgG (anti-TSH McAb) and BSA (in BSA–biotin conjugate) makes the two proteins tend to adsorb at different sites on the well surface. This not only reduces the adsorption competitiveness, but also weakens the denaturing effect of the IgG molecules, assuming that the closely arranged proteins can sustain each other to withstand the formation changes. Third, the four binding sites in the SA molecule and the mild reaction condition for biotinylating anti-T4 McAb also contributed to the improved activity of surface anti-T4 McAb.

Eu and Sm labeling

In order to get a high specific activity of the Sm-labeled BSA–T4 conjugate, the coupling of T4 to BSA was controlled to a relatively low level (3–5 T4 per BSA) to leave more $-NH_2$ available for Sm labeling. Moreover, the labeling reaction between BSA–T4 and the Sm labeling reagent was prolonged up to 2 days with a high excess of the Sm labeling reagents. Analysis indicated that the labeling ratio of BSA–T4 to Sm was ~27. Three anti-TSH McAbs that can form suitable sandwich pairs between each random couple were used to develop the present TSH/T4 TRFIA, of which McAb-05 was used as capture antibody and MbAc-03 and McAb-04 were labeled by Eu and used as detection antibodies [23]. The labeling ratios of McAb-03 and McAb-04 with Eu were about 8.0 and 11.2, respectively.

The kinetics

As shown in Figs. 6 and 7, the present TSH/T4 TRFIA showed rapid kinetics; the equilibrium of the reactions for both TSH and T4 assay could be reached within 30 min at 37 °C. The rapid agitation, small reaction volume, and high concentration of reaction reagent may have all contributed to this effect. In short, the immunoreactions in the present microwell-based TSH/T4 TRFIA occur mainly between the solid and the liquid interfaces; its kinetics displays pronounced diffusion dependence. The quick refreshing of the solution that closely approaches to the well surface, caused by the rapid vortex agitating, shortens the diffusion distance of the liquid ligand to its surface reaction partner,

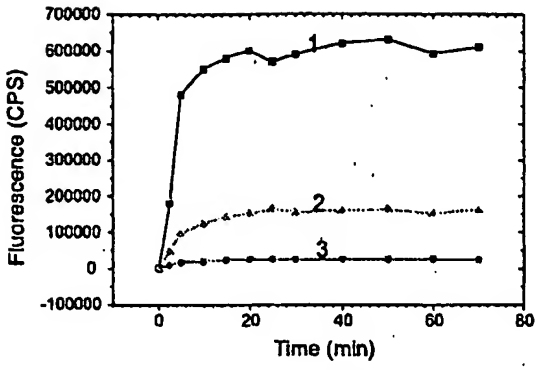

Fig. 6. Kinetics of the TSH assay in the present TSH/T4 TRFIA. Curves 1–3 were obtained by using standards F (TSH, 80.0 mIU/L), E (TSH, 19.0 mIU/L), and B (TSH, 0.21 mIU/L), respectively.

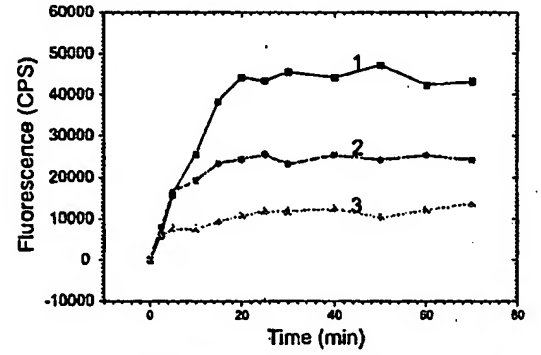

Fig. 7. Kinetics of the T4 assay in the present TSH/T4 TRFIA. Curves 1–3 were obtained by using standards F (T4, 0 nmol/L), D (T4: 50 nmol/L), and B (T4, 150 nmol/L), respectively.

thus shortening the reaction time for equilibrium. Because of the low nonspecific binding features of the labeled anti-TSH McAb and the T4–BSA conjugate (especially the former), we used high concentrations of labeled reagent for both the TSH and the T4 assay. The small reaction volume used in the assay further accelerated the reaction speed. The above factors make the reactions in TSH/T4 TRFIA faster than most of the reported DELFIA-based assays.

Detection of Eu and Sm fluorescence

Eu^{3+} or its chelate is the most commonly used label in time-resolved fluorometry-based analysis because Eu^{3+} complex often exhibits higher fluorescence quantum yield and lower background than other lanthanide complexes. At the same time, Sm^{3+}, Tb^{3+}, and Dy^{3+} can satisfy some analytical applications and are often chosen as a counterpart to europium for developing a multianalyte assay. The maximum emission of Sm^{3+} at 643 nm makes its fluorescence easy to distinguish from the interfering background. In the DELFIA enhancement solution, the considerable difference in decay time between the Eu^{3+} (~730 μs) and Sm^{3+} (~50 μs) fluorescence makes the temporal resolution of the two emission very efficient. The small Sm^{3+} emission peak at 598 nm does not extend to the normal Eu^{3+} window with an appropriate delay time (i.e., 400 μs). Compared to Sm^{3+}, Tb^{3+} chelates often haves longer decay time and higher fluorescence quantum yields, and their fluorescence is less sensitive to aqueous quenching. However, the relatively shorter emission wavelength of Tb^{3+} chelates (545 nm) makes them more prone to interference (e.g., phosphorescence) derived from plastic or glass materials; additionally, the fluorescence enhancement of Tb^{3+} in the DELFIA-type MAIA requires the use of an aliphatic β-diketone [26]. Considering the above factors, we selected Eu^{3+} and Sm^{3+} as labels to develop the present dual-label TSH/T4-TRFIA. In addition to the time and wavelength discriminations between the Eu/Sm signals, the following two additional means were adopted to suppress the minor interference arising from the signal spillover of the Eu/Sm labels. (1) An Eu^{3+}/Sm^{3+} normalization procedure that was set in the VICTOR^2 software was performed to correct the minor overlapping of the Eu/Sm fluorescence prior to detection. (2) The T4 and TSH concentrations in the standards were designed in opposite ways (low TSH concentration versus higher T4 concentration). The fluorescence of Eu^{3+} and Sm^{3+} in the first standard (TSH, 0 mIU/L; T4, 300 nmol/L) was rather low, generally 1500–3000 cps for Eu and 4000–7000 cps for Sm.

One problem encountered in developing DELFIA-type MAIA may relate to the relatively large difference in the excited energy levels of the ion labels used. As a consequence it is difficult to find a single organic ligand to enhance the fluorescence of all the ions at high efficiency. Wallac DELFIA enhancement solution, which was optimized for Eu detection, was used in the present TSH/T4 TRFIA. The lower quantum yield (~2%) and the shorter decay time (~50 μs) of the Sm chelate fluorescence in the DELFIA enhancement solution make the detection of Sm about 100 times less sensitive than that of Eu^{3+}, the lower limit of detection was $\sim 8.9 \times 10^{-14}$ mol/L for Eu^{3+} and $\sim 7.7 \times 10^{-12}$ mol/L for Sm^{3+} in our experiments. However, by employing the proposed cocoating strategy and the multiple Sm labeling of the BSA–T4 conjugate (~27 Sm/BSA–T4), the Sm^{3+} label was able to meet the requirements of T4 detection in the present TSH/T4 TRFIA. As a supplement to the DELFIA-type MAIA using lanthanide labels, recent investigations show that different metal atoms or ions (such as lanthanide) may act as a promising label alternative for developing MAIA when combined with a suitable mass spectrometry technique, e.g., the ICP-MS [24,27].

TSH/T4 TRFIA performances

The TSH/T4 TRFIA in this work is a solid-phase immunoassay based on the DELFIA technique and Eu/Sm labels. During the incubation, TSH molecules in the samples reacted with the Eu^{3+}-labeled anti-TSH antibody and the anti-TSH antibody immobilized on the well surface. In the meanwhile, T4 in the samples, which was released from its binding proteins by ANS and sodium salicylate, competes with the T4–BSA–Sm^{3+} conjugate for binding the limited amount of surface anti-T4 antibody. After washing, the unbound tracer was removed. The Eu^{3+} and Sm^{3+} on the bound immunocomplex (surface anti-TSH-antibody · TSH · anti-TSH-antibody-Eu^{3+} and surface anti-T4-antibody · T4–BSA–Sm^{3+}) were detected by time-resolved fluorescence measurement after being released by the enhancement solution in which they form highly fluorescent chelates.

Typical standard curves and the within-assay precision profiles of the present TSH/T4 TRFIA are shown in Figs. 4 and 5. The within-assay precision over the whole standard range was 4.2 to 8.5% for T4 assay and 2.6 to 5.2% for TSH assay. The standard curve for TSH detection can be extended up to ca. 800 mIU/L without hook effect. The lower limit of detection of the method was 0.028 mIU/L for TSH and 4.1 nmol/L for T4, estimated as the dose giving a signal of the mean of 10 replicates of zero dose plus (for TSH) or minus (for T4) 2 SD. It was noticed that the sensitivity of the TSH assay in present the method was simultaneously constrained by several factors. First, the blocking agent in the assay buffer used to dissociate T4 from its binding protein displays a significant inhibition of the immunoreactions between the TSH molecule and its antibody. Compared to the immunoreactions performed without the blocking agent, the blocking agent at the proposed concentration resulted in a signal decrease up to 30% or more. Second, although BSA–biotin used in the first cocoating step did not notably reduce the activity of surface anti-TSH McAb, the slight steric hindrance derived from the bound T4–BSA–Sm conjugate affected the sensitivity of TSH analysis to some extent (data not shown). Third, the low reaction volume required for rapid kinetics defined the sample amount and thus gave rise to a relatively low sensitivity of the TSH assay. Taking into account of the small sample volume used (20 µl), the absolute sensitivity (the detectable molecule number per test) of the TSH assay in the present TSH/T4 TRFIA was still obviously better than that of the well-optimized immunoradiometric assay and some of the sensitive nonisotopic label-based immunoassays [28,29].

The within- and between-run variations of the TSH/T4 TRFIA obtained with three serum samples are summarized in Table 1. Analytical recovery of the method was investigated by adding different amounts of TSH and T4 to a pooled serum. The original as well as the added amounts of TSH and T4 were measured with the TSH/T4 TRFIA. The recovery was 92.1–110.4% for TSH and 88.3–104.8% for T4. To study the linearity of the TSH/T4 TRFIA, three serum samples (TSH, 1.98–66.50 mIU/L; T4, 38.1–209.4 nmol/L) were serially diluted (1:2, 1:4, 1:8, 1:16, and 1:32) in hormone-free human serum and then measured with the TSH/T4 TRFIA. Both TSH and T4 assays in the TSH/T4 TRFIA gave good linearity (Table 2).

Table 1
Assay imprecision of the proposed TSH/T4 TRFIA

	Sample	Concentration	CV, % ($n = 12$)	
			Within-run	Between-run
TSH (mIU/L)	A	0.42	5.7	7.1
	B	1.65	2.5	4.6
	C	78.5	3.6	5.1
TT4 (nmol/L)	A	130.0	3.7	3.5
	B	90.0	3.5	6.1
	C	34.5	6.5	8.8

Table 2
Dilution test of the proposed TSH/T4 TRFIA

	Sample	Measurements under different dilution factors					
		Original	1:2	1:4	1:8	1:16	1:32
TSH (mIU/L)	1[a]	1.98	1.01	0.48	0.23	0.12	0.07
	2	4.30	2.12	1.10	0.49	0.26	0.12
	3[b]	66.50	31.06	16.25	8.80	4.21	2.17
TT4 (nmol/L)	1	209.4	102.5	49.2	27.4	12.1	/
	2	108.8	56.5	30.6	16.9	/	/

[a] Sample 1 was from a hyperthyroid patient with extraneous TSH added.
[b] Sample 3 with T4 value at ~38.1 nmol/L was not tested in the present dilution linearity study.

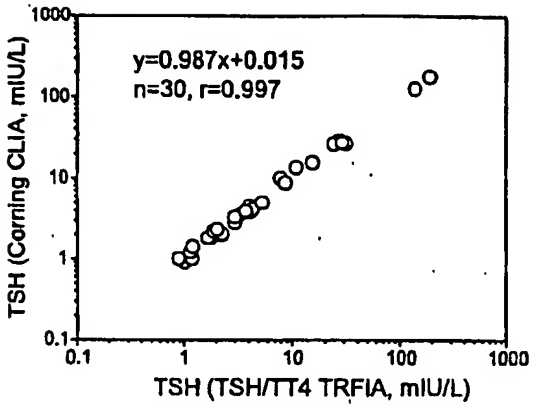

Fig. 8. Comparison of TSH in serum measured with the present TSH/T4 TRFIA and the Corning CLIA.

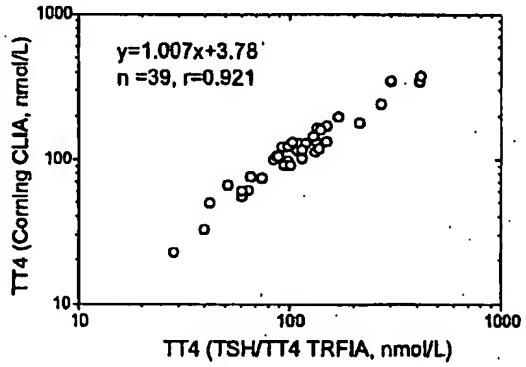

Fig. 9. Comparison of T4 in serum measured with the present TSH/T4 TRFIA and the Corning CLIA.

Correlation with CLIA

TSH and T4, in 30 and 39 clinical samples, respectively, were analyzed by the present TSH/T4 TRFIA. The correlation of the TSH values obtained by this method and those obtained by CLIA was excellent; the regression equation was CLIA = 0.987 TRFIA + 0.015 ($r = 0.997$). For T4, the regression equation was CLIA = 1.007 TRFIA + 3.78 ($r = 0.921$). The comparisons of TSH and T4 values obtained by the two methods (TSH/T4 TRFIA and CLIA) are shown in Figs. 8 and 9.

Conclusion

In summary, we herein described a sensitive, reproducible, and accurate TRFIA for simultaneous detection of human serum TSH and T4. As TSH and T4 could be measured in a single assay, the overall procedure became simple and the hands-on time was short. In order to achieve sensitive TSH detection and make the Sm signal strong enough for T4 assay, a novel cocoating strategy was proposed to prepare highly active surface anti-TSH and anti-T4 antibody.This cocoating method is believed to be applicable to various other kinds of molecules, especially molecules with high hydrophilicity or with low molecular weight that cannot be effectively immobilized by simple passive adsorption. The ready commercial availability of various biotinylation reagents with different functional groups makes the cocoating strategy more valuable, because the method could potentially be used to attach any molecules with a high efficiency provided that the target molecule can be biotinylated. The data presented here indicate that rapid kinetics can be obtained with regard to the DELFIA-type and microwell-based TRFIA due to the intrinsically high sensitivity of the DELFIA technique and the small hydrophilic label that enables using high amounts of labeled reagents.

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